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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classificati n 5 :		(11) International Publication Number:	WO 92/07943
C12N 15/63, 15/86, 15/67 C12N 15/12, 7/01	A1	(43) International Publication Date:	14 May 1992 (14.05.92)

(21) International Application Number: PCT/US91/08121

(22) International Filing Date: 31 October 1991 (31.10.91)

(30) Priority data:
607,252 31 October 1990 (31.10.90) US

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(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU+,TD (OAPI patent), TG (OAPI patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: RETROVIRAL VECTORS USEFUL FOR GENE THERAPY

(57) Abstract

Retroviral vectors are disclosed which include an insertion site for genes of interest and are capable of expressing high levels of the protein derived from the genes of interest in a wide variety of transfected cell types. Also disclosed are retroviral vectors lacking a selectable marker, thus rendering them suitable for human gene therapy in the treatment of a variety of disease states without the co-expression of a marker product, such as an antibiotic. These retroviral vectors are especially suited for use in certain packaging cell lines.

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RETROVIRAL VECTORS USEFUL FOR GENE THERAPY

The present invention is directed to novel retroviral vectors capable of being used in somatic gene therapy. These retroviral vectors include an insertion site for the genes of interest and are capable of expressing controlled levels of the protein derived from the genes of interest in a wide variety of transfected cell types. One class of novel retroviral vectors of the invention lacks a selectable marker, thus rendering them suitable for human somatic therapy in the treatment of a variety of disease states without the co-production of an marker gene products, such as antibodies. The retroviral vectors of the invention are especially suited for use in certain packaging cell lines.

Background

Numerous methods exist for genetically engineering mammalian cells. There is great interest in genetically engineering mammalian cells for several reasons including the need to produce large quantities of various polypeptides and the need to correct various genetic defects in the cells. The methods differed dramatically from one another with respect to such factors as efficiency, level of expression of foreign genes, and the efficiency of the entire genetic engineering process.

One method of genetically engineering mammalian cells that has proven to be particularly us ful is by means of retroviral vectors. Retrovirus vectors and their uses are described in many publications including Mann, et al., Cell 33:153-159 (1983) and Cone and Mulligan, Proc. Natl. Acad. Sci. USA 81:6349-

6353 (1984). Retroviral vectors are produced by genetically manipulating retrovirus s.

Retroviruses are RNA viruses; that is, the viral genome is RNA. This genomic RNA is, however, reverse transcribed into a DNA copy which is integrated stably and efficiently into the chromosomal DNA of transduced cells. This stably integrated DNA copy is referred to as a provirus and is inherited by daughter cells as any other gene. As shown in Figure 1, the wild type retroviral genome and the proviral DNA have three Psi genes: the gag, the pol and the env genes, which are flanked by two long terminal repeat (LTR) sequences. The gag gene encodes the internal structural (nucleocapsid) proteins; the pol gene encodes the RNA directed DNA polymerase (reverse transcriptase); and the env gene encodes viral envelope glycoproteins. The 5′ and 3′ LTRs serve to promote transcription and polyadenylation of virion RNAs.

Adjacent to the 5′ LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the Psi site). Mulligan, R.C., In: Experimental Manipulation of Gene Expression, M. Inouye (ed), 155-173 (1983); Mann, R., et al., Cell, 33:153-159 (1983); Cone, R.D. and R.C. Mulligan, Proceedings of the National Academy of Sciences, U.S.A., 81:6349-6353 (1984).

If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the result is a cis acting defect which prevents encapsidation of genomic RNA. However, the

r sulting mutant is still capable of directing the synthesis of all virion proteins. Mulligan and coworkers have described retroviral genomes from which these Psi sequences have been deleted, as well as cell lines containing the mutant genome stably integrated into the chromosome. Mulligan, R.C., In Experimental Manipulation of Gene Expression, M. Inouye (ed), 155-173 (1983); Mann, R., et al., Cell, 33:153-159 (1983); Cone, R.D. and R.C. Mulligan, Proceedings of the National Academy of Sciences, U.S.A., 81:6349-6353 (1984). Additional details on available retrovirus vectors and their uses can be found in patents and patent publications including European Patent Application EPA 0 178 220, U.S. Patent 4,405,712, Gilboa, Biotechniques 4:504-512 (1986) (which describes the N_2 retroviral vector). The teachings of these patents and publications are incorporated herein by reference.

Retroviral vectors are particularly useful for modifying mammalian cells because of the high efficiency with which the retroviral vectors "infect" target cells and integrate into the target cell genome. Additionally, retroviral vectors are highly useful because the vectors may be based on retroviruses that are capable of infecting mammalian cells from a wide variety of species and tissues.

The ability of retroviral vectors to insert into the genome of mammalian cells have made them particularly promising candidates for use in the genetic therapy of genetic diseases in humans and animals. Genetic therapy typically involves (1) adding new genetic material to patient cell in vivo, or (2) r moving patient

cells from the body, adding new genetic material to the cells and reintroducing them into the body, i.e., in vitro gene therapy. Discussions of how to perform gene therapy in variety of cells using retroviral vectors can be found, for example, in U.S. Patent Nos. 4,868,116, issued September 19, 1989, and 4,980,286, issued December 25, 1990 (epithelial cells), WO89/07136 published August 10, 1989 (hepatocyte cells), EP 378,576 published July 25, 1990 (fibroblast cells), and WO89/05345 published June 15, 1989 and WO/90/06997, published June 28, 1990 (entothelial cells), the disclosures of which are incorporated herein by reference.

In order to be useful for the various techniques of gene therapy, suitable retroviral vectors require special characteristics that have not hither to been available. A primary source of the need for these special requirements of the vector for use in the in vivo genetic manipulation of patient cells in gene therapy is because it is usually not feasible to use retroviral vectors that require a selection for integration of the vector into the genome of "patient" cells. For example, typical retroviral vectors, e.g., MSV DHFR-NEO described in Williams, et al., Nature 310:476-480 (1984), uses neomycin resistance as a suitable marker for detecting genetically modified cells. Thus, with such neomycin resistant retroviral vectors, patients would be required to be exposed to high levels of neomycin in ord r to effect genetic repair of cells through in vivo gene therapy. Moreover, in both in vivo and in vitro gene therapy it may

be undesirable to produce the gene product of the marker gene in cells undergoing human g ne somatic therapy. For example, there is no therapeutic reason to produce large levels of neomycin phosphotransferase in blood cells undergoing hemoglobin gene replacement for curing a thalassemia. Therefore, it would be desirable to develop retroviral vectors that integrate efficiently into the genome, express desired levels of the gene product of interest, and are produced in high titers without the co-production or expression of marker products such as antibodies.

Summary of the Invention

The present invention is directed to novel retroviral vectors capable of being used in somatic gene therapy. These retroviral vectors include an insertion site for the genes of interest and are capable of expressing desired levels of the protein derived from the gene of interest in a wide variety of transfected cell types.

In one aspect of the invention there is provided a retroviral vector comprising in operable combination, a 5′ LTR and a 3′ LTR derived from a retrovirus of interest, and an insertion site for a gene of interest, and wherein at least one of the gag, env or pol genes in the vector are incomplete or defective. The vector preferably contains a splice donor site and a splice acceptor site, wherein the splice acceptor site is located upstr am from the site where the gene of interest is inserted. Also, the vector desirably contains a gag

transcriptional promoter functionally positioned such that a transcript of a nucleotide sequenc inserted into the insertion site is produced, and wherein the transcript comprises the gag 5' untranslated region. The preferred vectors of the invention are lacking a selectable marker, thus, rendering them more desireable in human somatic gene therapy because a marker gene product, such as an antibiotic drug marker will not be co-produced or co-expressed.

The gene of interest that is incorporated in the vectors of the invention may be any gene which produces a hormone, an enzyme, a receptor or a drug(s) of interest.

The retroviral vectors are most suitably used in combination with certain packaging cells, as herein defined, which in turn may be used in a wide variety of cell types for human or animal somatic gene therapy.

A particular prefered retroviral vector of the invention is identified herein as "MFG", as depicted in Figures 2c and 3, and the plasmid containing it, and especially the plasmid MFG having the indentifying characteristics of ATCC No. 68,754.

The present invention is also directed to retroviral vectors similar to those described above, but further comprising a non-LTR enhancer and the alpha-globin transcriptional promoter sequence in order to control the expression of various genes of interest. This aspect of the invention specifically provides for the use of an enhancer sequence from cytomegalovirus. Also provided for are vectors in which the enhanc r sequence is deleted from the 3' LTR thus resulting in the inactivation of the 5' LTR upon

integration of the vector into the genome. Th α-globin promoter containing vector α-SGC is specifically provided, and especially that which is depicted in Figure 4, and the plasmid containing it, and especially the plasmid α-SGC having the identifying characteristics of ATCC No. 68,755.

The subject invention also includes retroviral vectors that have a gene for expression inserted into the site for gene expression. Specific examples include MFG and α-SGC vectors containing the genes for human factor VIII or tPA inserted into the site for expression of these useful products.

Description of Drawings

Figure 1 is a schematic representation of a wild type murine leukemia virus (retroviral) genome.

Figure 2 is a schematic representation of retroviral vectors, each having a recombinant genome, useful in the present invention. Figure 2a is pLJ and Figure 2b is pEm, Figures 2c is MFG and Figure 2D is α-SGC.

Figure 3 is a schematic diagram of the retroviral vector MFG.

Figure 4 is a schematic diagram of the retroviral vector α-SGC.

Figure 5 is a histogram showing the potency after implantation into dogs of synthetic grafts lined with endothelial cells genetically augmented to express tPA.

Figure 6 is a diagram of the factor VIII polypeptide. Figure 6b is a diagram of the factor VIII cDNA showing the restriction enzyme sites used in the various constructs to generate

the retroviral vector. Figure 6c is a diagram of the deletion derivative of the factor VIII cDNA inserted into the retroviral vector with the deleted region shown as vertical lines. Figure 6d is an expanded diagram of the B domain deletion between the Hind III and Pst I sites. The nucleotide sequence at the junction of the heavy chain and light chain is denoted above the line and the corresponding amino acid numbers are denoted below the line.

Figure 7 is a diagram of the assembled final retroviral vector, MFG-factor VIII.

Figure 8 is a diagram of the α-SGC-LacZ recombinant retrovirus.

Figures 9(a) and 9(b) represents a schematic diagram of the construction of the MFG vector of the invention.

Figure 10 is a schematic representation of the modification of the tPA gene, the oligonucleotides used to facilitate the modification and the insertion of the modified tPA gene into the MFG vector.

Description of Specific Embodiments

The subject invention provides for several retroviral vectors. The retroviral vectors provided for contain (1) 5′ and 3′ LTRs derived from a retrovirus of interest, the preferred retrovirus source for the LTRs is the Maloney murine leukemia virus, and (2) an insertion site for a gene of interest. The retrovirus vectors of the subject invention do not contain either a complete gag, env, or pol gene, so that the retroviral vectors are incapable of independent replication in target cells. Preferred retroviral vectors contain a portion of the gag

coding sequence, preferably the partial gag coding sequence comprises a splice donor site and a splice acceptor site, positioned such that the partial gag sequence is located in the retroviral vector so that the splice acceptor site is located closest to, and upstream from, the insertion site for the gene of interest. In a particularly preferred embodiment of the subject vectors, the gag transcriptional promoter is positioned such that a transcript initiated from the gag promoter contains untranslated 5′ gag sequence and transcript produced from nucleic acid sequence inserted into the insertion site in the vector. Vectors of interest preferably do not contain selectable markers. A preferred embodiment of such vectors is the vector designated as "MFG".

Another aspect of the subject invention is to provide for retroviral vectors lacking functional enhancer elements in the 3′ LTR, thereby inactivating the 5′ LTR upon integration into the genome of target organisms.

Another aspect of the subject invention is to provide for retroviral vectors essentially as described above but instead of utilizing the gag promoter to control the expression of a gene inserted into the insertion site of the vector, a human alpha-globin gene transcriptional promoter is used. The retroviral vector α-SGC is specifically disclosed.

Another aspect of the subject invention is to employ enhancer sequences not located in the LTRs in r troviral vectors using the alphaglobin transcriptional promoter to increase the expression of a gene of interest. Of particular inter st are vectors in which the enhancer

sequ nce is placed upstream of the alpha-globin transcriptional promoter. Another aspect of the subject invention is to the enhancer sequence derived from a cytomegalovirus in such non-LTR enhancer containing vectors.

Another aspect of the subject invention is to provide for retrovirus vector constructions containing genes for expression inserted into the insertion site in the retrovirus vector. Genes for insertion into the subject retrovirus vectors include any of a variety of hormones, enzymes, receptors or other drugs. The subject invention specifically provides for the genetic contructions consisting of tPA and Factor VIII inserted (individually) into the insertion, i.e., cloning sites of MFG and α-SGC.

The wild type retroviral genome has been modified by Cone and Mulligan, supra for use as a vector capable of introducing new genes into cells. As shown in Figures 2, the gag, the pol and the env genes have all been removed and a DNA segment encoding the neo gene has been inserted in their place. The neo gene serves as a dominant selectable marker. The retroviral sequence which remains part of the recombinant genome includes the LTRs, the tRNA binding site and the Psi packaging site. Cepko, C. et al., Cell, 37:1053-1062 (1984).

In addition to teaching numerous retroviral vectors containing sites for insertion of foreign genes for expression, the subject invention also provides for genetic constructions in which the retroviral vectors contain genes inserted into the site for insertion i.e., foreign genes or genes for expression. Foreign genes for inclusion in the

vectors of the subject invention may ncode a variety of proteins. Proteins of interest include various hormones, growth factors, enzymes, lymphokines, cytokines, receptors and the like. The term "foreign genes" includes nucleic acid sequences endogenous to cells into which the retrovirus vector containing the foreign gene may be inserted. Of particular interest for use as genes for expression are those genes encoding polypeptides either absent, produced in diminished quantities, or produced in mutant form in individuals suffering from a genetic disease. Additionally, it is of interest to use foreign genes encoding polypeptides for secretion from the target cell so as to provide for a systemic effect by the protein encoded by the foreign gene. Specific foreign genes of interest include those encoding hemoglobin, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, etc., GM-CSF, G-CSF, M-CSF, human growth factor, insulin, factor VIII, factor IX, tPA, LDL receptors, tumor necrosis factor, PDGF, EGF, NGF, IL-1ra, EPO, β-globin and the like, as well as biologically active muteins of these proteins. Genes for expression for insertion into retroviral vectors may be from a variety of species; however, preferred species sources for genes of interest are those species into which the retroviral vector containing the foreign gene of int rest is to be inserted.

The retroviral vectors of the subj ct invention are typically used by transfecting the nucleic acid s qu nces into packaging c ll

lines. Packaging cell lines contain viral g ne functions that have been deleted from the retrovirus in course of converting it to a vector. Thus transfecting retroviral vectors of the subject invention, either with or without genes for expression inserted into the vector insertion site, into packaging cell lines results in the production of infectious virus particles containing the desired genetic construction. Ideally, packaging cell lines are capable of producing a high titerl of recombinant retrovirus. Preferred packaging cell lines for use with the genetic constructions of the subject are Psi2, Psi-Am, Psi CRIP, and Psi CRE. Psi2 is particularly preferred for use with the retroviral vectors MFG and α-SGC.

The Psi 2 cell line described by Mulligan and coworkers was created by transfecting NIH 3T3 endothelial cells with pMOV-Psi, which is an ecotropic Moloney murine leukemia virus (Mo-MuLV) clone. pMOVPsi expresses all the viral gene products but lacks the Psi sequence, which is necessary for encapsidation of the viral genome. pMOV-Psi- expresses an ecotropic viral envelope glycoprotein which recognizes a receptor present only on mouse (and closely related rodent) cells.

Another cell line is the Psi am line, which are Psi-2-like packaging cell lines. These Psi-am cell lines contain a modified pMOV-Psi-genome, in which the ecotropic envelope glycoprotein has been replaced with envelope sequences derived from the amphotropic virus 4070A. Hartley, J.W. and W.P. Rowe, Journal of Virology, 19: 19-25 (1976) . As a result, they

are useful for production of recombinant virus with amphotropic host range. The retrovirus used to make the Psi am cell line has a very broad mammalian host range (an amphotropic host range) and can be used to infect human cells. If the recombinant genome has the Psi packaging sequence, the Psi-am cell line is capable of packaging recombinant retroviral genomes into infectious retroviral particles. Cone, R. and Mulligan, R.C. Proceedings of the National Academy of Sciences, USA, 81:6349-6353 (1984).

Two other packaging cell lines are known as Psi CRIP and Psi CRE. These cell lines have been shown to be useful to isolate clones that stably produce high titers of recombinant retroviruses with amphotropic and ecotropic host ranges, respectively. These cell lines are described in Danos, O. and R.C. Mulligan, Proceedings of the National Academy of Sciences, USA, 85: 6460-6464 (1988) and in U.S. patent application Serial No. 07/239,545 filed September 1, 1988. The teachings of the reference and the patent application are incorporated herein by reference. Psi CRIP and Psi CRE have been deposited at the American Type Culture Collection, Rockville, MD, under Accession Nos. CRL 9808 and CRL 9807, respectively, under the terms of the Budapest Treaty.

The retroviral vectors of the invention may be used in a wide variety of cell types, including without limitation, epithelial cells, fibroblast cells, heptocyte cells, endothelial cells, myoblast cells, astrocyte cells, lymphocyte cells, m senthial cells, and the like. Of particular interest are the cell types disclosed in the following patents and patent publications

U.S. Patent Nos. 4,868,116, issued Septemb r 19, 1989, and 4,980,286, issued December 25, 1990 (epithelial cells), PCT/US89/00422, WO89/07136 published August 10, 1989 (hepatocyte cells), EP 378,576 published July 25, 1990 (fibroblast cells), and PCT/US88/o4383, WO89/05345 published June 15, 1989 and WO/90/06997, published June 28, 1990 (entothelial cells), the disclosures of which are incorporated herein by reference.

The vectors of the subject invention find a variety of uses in the treatment of various medical conditions including, without limitation cancer, genetically based diseases, cardiopulmonary diseases, endocrinological diseases, and the like.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

In Examples 1(a)-(c) there is described the precursor elements for constructing the MFG vector of the invention. The citations for the references are herein incorporated by reference.

Example Ia

Construction of Vectors

pMOV. (pMOVPsi) was constructed as follows: Three purified DNA fragments were ligated together to construct pMOV Psi-. The first was obtained by digesting pMOV Psi+ with Xho I to completion, followed by partial digestion with EcoRI. Chumakov, I. et al., , Journal of Virology, 42:1088-1098 (1982). The fragment extending from the Xho I site at 2.0 U in MuLV, through the 3' LTR, 3' mouse flanking sequence, all of pBR322, and ending at the EcoRI site was purified from an agarose gel after

el ctrophoretic separation. Vog lstein, B. and D. Gillespie, Proceedings of the National Academy of Sciences, USA, 761:615-619 (1979). The second fragment was obtained by digestion of pMOV Psi+ with Bal I to completion followed by purification of the fragment extending from the Bal I site in pBR322 through 5' mouse flanking sequence and 5' LTR to the Bal I site located at 0.7 U of MuLV. HindIII linkers (Collaborative Research) were then blunt-ligated to this fragment with T4 DNA ligase, and the fragment was digested with excess HindIII and EcoRI. The LTR- containing fragment was purified from an agarose gel after electrphoretic separation. The third fragment present in the final ligation reaction was obtained from pSV2gag/pol where the gag/pol region of MuLV had been subcloned into pSV2. Mulligan, R.C. and P. Berg, Science, 209:1422-1427 (1980). pSV2- gag/pol was digested to completion with Xho I and HindIII and the fragment extending from the HindIII site (changed from the Pst I site at 1.0 U of MuLV) to the Xho I site at 2.0 of MuLV was purified from an agarose gel following electrophoretic separation. These three DNA fragments were then mixed in equimolar amounts at a total DNA concentration of 50 ug/ml. in ligase buffer (50 mM Tris-HCl [pH 7.8], 10 mM $MgCl_2$, 20 mM dithiothreitol, 1.0 mM ATP, 50 ug/ml. bovine serum albumin) and incubated with T4 DNA ligase for 18 hr. at 15 C. E. coli HB101 was transfected with the ligated DNA, and ampicillin resistant transfectants were obtained. The plasmid DNA obtained from a number of transformants was screened for the desir d structure by digestion with appropriate

restriction endonucleases and el ctrophoresis through agarose gels. Davis, R.W. et al., Methods in Enzymology, 65:404-411 (1980).

Cell lines containing the Psi mutant stably integrated into the chromosome were made by cotransfection of pMOV-Psi and pSV2gpt, a SV40 hybrid vector capable of XG PRT expression. Mulligan, R.C. and P. Berg, Science, 209:1422-1427 (1980). Cells from gpt+ colonies obtained in this way were cloned and established into three lines: Psi-1, Psi-2, and Psi-3.

Example 1(b)

pLJ. The characteristics of this vector have been described in Korman, A.J. et al., Proceedings of the National Academy of Sciences, USA, 84:2150 (1987) . This vector is capable of expressing two genes: the gene of interest and a dominant selectable marker, such as the neo gene. The gene of interest is cloned in direct orientation into a BamHI/SmaI/SalI cloning site just distal to the 5′ LTR, while, the neo gene is placed distal to an internal promoter (from SV40) which is farther 3′ than is the cloning site (is located 3′ of the cloning site). Transcription from pLJ is initiated at two sites: 1) the 5′ LTR, which is responsible for expression of the gene of interest and 2) the internal SV40 promoter, which is responsible for expression of the neo gene. The structure of pLJ is represented in Figure 2a.

Vector pLJ is represented in Figure 2a. In pLJ, the genetic material of interest is inserted just following the 5′ LTR. Expression of this g n tic material is transcribed from the LTR and expression of the neo gene is

transcribed from an internal SV40 promoter.

Example I(c)

pEm. In this simple vector, the entire coding sequence for gag, pol and env of the wild type virus is replaced with the gene of interest, which is the only gene expressed. The components of the pEm vector are described below. The 5' flanking sequence, 5' LTR and 400 bp of contiguous sequence (up to the BamHI site) is from pZIP. The 3' flanking sequence and LTR are also from pZIP; however, the ClaI site 150 bp upstream from the 3' LTR has been ligated with synthetic BamHI linkers and forms the other half of the BamHI cloning site present in the vector. The HindIII/EcoRI fragment of pBR322 forms the plasmid backbone. This vector is derived from sequences cloned from a strain of Moloney Murine Leukemia virus. An analogous vector has been constructed from sequences derived from the myeloproliferative sarcoma virus. The structure of pEm is represented in Figure 2b.

Vectors without a selectable marker can also be used to transduce a variety of cell types, such as endothelial cells with genetic material of interest. Such vectors are basically simplifications of the vectors previously described, in which there is such a marker. Vector pEm is represented in Figure 2b; as represented, the main components of the vector are the 5' and 3' LTR, and the genetic material of interest, inserted between the two LTRs.

Example II

Construction of the MFG Vector

The MFG vector having the id ntifying

characteristics of ATCC accession No. 68754 is derived from the pEM vector but contains 1038 base pairs of the gag sequence from MMLV to increase the encapsulation of recombinant genomes in the packaging cell lines, and 350 base pairs derived from MOV-9 which contains the splice acceptor sequence and transcriptional start. An 18 base pair oligonucleotide containing NcoI and BamHI sites directly follows the MOV-9 sequence and allows for the convenient insertion of genes with compatible sites. The MMLV LTR controls transcription and the resulting mRNA contains the authentic 5' untranslated region of the native gag transcript followed directly by the open reading frame of the inserted gene. The structure of MFG is represented in Figure 2c. A more detailed map of MFG is provided in Figure 13. Details for the construction of MFG are provided in Figures 9(a) and 9(b).

MFG was constructed by ligating the 5' LTR containing XhoI/NdeI fragment of the half-GAG retroviral vector (half-GAG is described in Bender, et al., J. Virol. 6I:1639-1646) to an XhoI/BamHI H4 histone promoter fragment. Retroviral vector pEM was digested with NdeI and BamHI, and the 3' LTR containing fragment was ligated to the halfGAG fragment already ligated to the H4 fragment so as to produce an intermediate retrovirus vector containing 2 LTRs in the proper orientation and also containing the H4 fragment within the viral portion of the vector. The intermediate vector was then linearized by digestion with NdeI and the NdeI site in the pB322 portion of the vector was filled in by polymerase and destroyed by

ligation. The vector was subsequently digest d with XhoI and the XhoI site was joined to an NdeI linker. The vector was subsequently cleaved with BamHI and the large fragment containing both LTRs and the pBR322 sequence) was purified.

A linker having XhoI and BamHI and having the following sequence:

CTAGACTGCCATGGCGCG
TGACGGTACCGCGCCTAG

was synthesized and ligated to both the BamHI site on the cleared intermediate vector and an NdeI/XbaI fragment from pMOV9 [containing a splice acceptor site next to the NdeI edge] so as to form a circular vector, MFG as illustrated in Figures 2c, 3 and 9(a) to 9(b). The a plasmid containing vector MFG has been deposited with the American Type Culture Collection and it has accession number 68,754.

Example III

Construction of α-SGC

The α-SGC vector (ATCC accession number 68755) utilizes transcriptional promoter sequences from the α-globin gene to regulate expression of the tPA gene. The 600 base pair fragment containing the promoter element additionally contains the sequences for the transcriptional initiation and 5′ untranslated region of the authentic α-globin mRNA. A 360 base pair fragment which includes the transcriptional enhancer from cytomeglovirus precedes the α-globin promoter and is used to enhance transcription from this element. Additionally, the MMLV enhancer is deleted from the 3′ LTR. This deletion is transferr d to the 5′ LTR upon infection and essentially inactivates the transcriptional activating activity of the element. The structure of α-SGC

is represented in Figure 2d. A more detailed description of αSGC is provided in Figure 4. A plasmid containing the α-SGC vector has been deposited with the American Type Culture Collection and it has accession number 68,755.

The following examples provide examples of using the retroviral vectors of the invention using endothelial cells. It will be understood that other cell types are suitable as well, including without limitation epithelial cells, fibroblast cells, heptocyte cells and others.

EXAMPLE IV Increased Expression of tPA by Genetically Modified Canine and Human Endothelial Cells

Tissue plasminogen activator (tPA) is a protein normally secreted by endothelial cells that promotes fibrinolysis of blood clots. Recombinant retroviral vectors encoding human tPA were constructed and used to transduce canine endothelial cells in order to demonstrate the enhanced delivery of a therapeutically relevant protein from transduced endothelial cells.

The modifications of the tPA gene for cloning into the recombinant retroviral vectors are shown in Figure 10. The coding sequences of human uterine tPA were contained within a Sal I DNA fragment of a pUC-based plasmid obtained from Integrated Genetics Inc. Framingham MA. The Sal I fragment was derived by placing Sal I linkers at the SFaN I site at base pair 6 and the Bgl II site at base pair 2090 of the original cDNA. The coding sequences extends from base pair 13 to base pair 1699.

From this original clone a fragment that could be cloned directly into the MFG and a-SCG vectors described in the body of this patent was derived. The Sal I fragment was first converted to a Bam HI fragment by the addition of synthetic Bam HI linkers and then digest with the restriction enzyme Bgl II to yield a 109 base pair BamHI to BglII fragment and a 1975 base pair Bgl II to Bam HI fragment. To recreate the missing 100 base pairs of tPA coding sequences and the translational start codon, two 104 base pair oligonucleotides were chemically synthesized and annealed to create a fragment with an Nco I site at the 5′ end and a Bgl II site at the 3′ end. This oligonucleotide was ligated onto the Bgl II site of the partial 1975 base pair tPA gene to create a 2079 base pair tPA gene with the identical coding sequence of the original molecule, but which can be easily obtained as an Nco I to Bam HI fragment. It was inserted directly into the MFG and α-SGC vectors (the resulting vectors were given ATCC accession numbers 68727 and 68729, respectively). These manipulations were performed by standard molecular biological techniques (Molecular Cloning -A laboratory Manual, T. Maniatis, E.F. Frisch, and J. Sambrook), and are diagrammed in Figure 2.

Cell lines producing recombinant virus encoding MFG-tPA and α-SGC-tPA were made from the Psi packaging cell line of Danos and Mulligan capable of producing recombinant retrovirus of amphotrophic host range [Proc. Natl. Acad. Sci. U.S.A. 85:6460 (1988)]. 10 ug of the specified DNAs and 1 ug of the plasmid pSV2neo were co-precipitated and transfected

onto the packaging cells by standard calcium phosphate transfection procedures. Stably transfected clones were isolated after growth for 14 days in selective media containing 800 ug/ml G418. 24 hour culture supernatants were obtained from confluent monolayers of individual clones and used to infect NIH 3T3 cells. The culture supernatants were removed after 24 hours exposure, and the 3T3 cells were refed with normal media and allowed to grow for an additional 72 hours. Fresh media was placed on these cells for 6 hours and these supernatants were assayed for human tPA with a commercially available ELISA specific for human tPA (Immunobind-5, American Diagnostica Inc., N.Y., N.Y.) From this screen, clones of the packaging cell line producing either the MFG-tPA recombinant virus or the α-SGC-tPA recombinant virus were selected and designated MFG 68 and α-SGC 22, respectively.

Canine endothelial cells were isolated from 10 cm segments of the external jugular vein by collagenase digestion as described [T.J. Hunter, S.P. Schmidt, W.V. Sharp, and (1983) Trans. Am. Soc. Artif. Intern. Organs 29:177]. The cells were propagated on fibronectin-coated tissue culture dishes in M199 media containing 5% plasma-derived equine serum, 50 ug/ml endothelial cell growth factor, and 100 ug/ml heparin. Purity of the cell cultures was determined by immunohistochemical assay for the presence of Von Willebrands Factor and the absence of smooth muscle cell specific α-actin. The day before transduction, the endothelial cells were seeded at 5.5 X 10^3 cells/cm^2 in medium without heparin. The following day, the

ndoth lial cells w re exposed for 24 hours to supernatants containing recombinant virus derived from each producer cell line to which was added 8 ug/ml polybrene. The viral supernatants were removed, the cells feed with normal media and growth was allowed to proceed for an additional 48 hours before analysis.

High molecular weight genomic DNA and total RNA were isolated from cultures of endothelial cells by standard techniques (Molecular Cloning-A Laboratory Manual, T. Maniatis, E.F. Fritsch, and J. Sambrook). The DNA and RNA were analyzed by hybridization analysis with a ^{32}P-labeled DNA probe prepared from the entire tPA cDNA fragment. Standard techniques were used for electrophoretic separation, filter transfer, hybridization, washing, and ^{32}P-labeling (Molecular Cloning-A Laboratory Manual T. Maniatis, E.F. Fritsch, and J. Sambrook) . The production of human tPA in transduced canine endothelial cells was demonstrated with a species specific immunocytochemical stain. Transduced cells were fixed in 3% formaldehyde for 10 minutes at room temperature and then permeabilized in 0.1% Triton X-100 for 5 minutes. The fixed cell monolayer was then incubated sequentially with a murine monoclonal antibody to human tPA, with an alkaline phophatase conjugated goat anti-mouse antibody, and finally with a color reagent specific for alkaline phophatase. This procedure specifically stains those cells expressing human tPA and can be visualized by conventional light microscopy. In addition, tPA secretion from transduced cells was determined from confluent cell monolayers. Fresh media was placed on the cells for 6 hours,

removed and clarified by centrifugation, and the amount of human tPA determined with a commercially available ELISA (Immunobind-5, American Diagnostica).

The efficiency of the transduction process is shown by immunocytochemical stain of a population of cells mock transduced or transduced with MFG-tPA. As shown in Figure 5, after a single exposure of the cells to a viral supernatant harvested from MFG 68, essentially all of the cells are synthesizing human tPA as opposed to none of the cells in the control. This was achieved without selection of any type for transduced cells.

An immunological assay was conducted to determine the amount of tPA that was being secreted from transduced cultures. As shown below, cells transduced with recombinant virus from either MFG 68 or α-SGC 22 secreted large amounts of human tPA. Under similar conditions, human endothelial cells in culture typically secrete approximately 1 ng of tPA [Hanss, M., and D. Collen (1987) J. Lab. Clin. Med. 109: 97-104].

TABLE I

Cells		ng human tPA/million cells/6 hours
uninfected	K9 EC	0.0
MFG 68	K9 EC	150.1
α-SGC 22	K9 EC	302.8

As a further confirmation that the endothelial cells had been transduced with recombinant virus

from MFG 68 and α-SGC 22, DNA and RNA was isolated from transduced cells and analyzed by hybridization to a radiolabeled tPA gene. An autoradiogram of the DNA analysis was performed. No hybridization was detected in the uninfected controls, but single hybridizing species of the appropriate molecular weight was seen in the cells infected with the two recombinant vectors. This demonstrates that the genetic information has been transferred to the genome of these transduced cells.

Hybridization analysis of total RNA isolated from these cells confirms the protein and DNA results. Again no hybridization was detected in the control cells but in the RNA derived from the transduced cells hybridizing bands of the appropriate sizes can be seen. RNA from the MFG 68 and α-SGC 22 recombinant virus producing cells is also shown as controls.

EXAMPLE V In vivo Function of Transduced Canine Endothelial Cells Transplanted on the Surface of Vascular Grafts

Endothelial cells were enzymatically harvested from external jugular veins of adult female mongrel dogs that weighed 20-25 kg and cultured in the laboratory and analyzed for purity as described in Example IV. One half of the cells isolated from each animal were transduced by two exposures to supernatants harvested tPA from the MFG 68 cell line producing the MFG-tPA recombinant virus as described in the previous section. The oth r half were mock transduced. Growth curves conducted on ach population

showed no difference in growth characteristics. ELISA measurements were made on culture supernatants derived from each batch of transduced cells to assure that tPA was being secreted from the augmented cells. These cells were then propagated in the laboratory for approximately one week to obtain sufficient numbers of cells.

For each animal from which cells had been isolated, two vascular grafts made of expanded Teflon (W.L. Gore and Associates, Inc. Flagstaff, AZ) were seeded with cells. One graft was seeded with mock transduced cells, and the other with cells transduced to secrete high levels of tPA. Each graft, measuring 0.4 cm x 14 cm, was precoated with 1.5 ug/cm^2 fibronectin (Sigma Chemical Corp., St. Louis MO) , and then seeded with 2200,000 endothelial cells/cm. The grafts were then incubated for an additional 72 hours in culture. Prior to implant the ends were cut off each graft and checked to assure cell coverage.

The same dogs from which the cells had been harvested were anesthetized and 10 cm segments of the seeded grafts were implanted as aorta-iliac bypasses. Each dog received two contralateral grafts; one seeded with control cells and the other seeded with cells that had been transduced to secrete high levels of tPA. Following implantation the performance of the grafts was monitored daily with a B-mode scanner which locates the graft with ultrasound and assesses blood flow through the graft by Doppler measurements (Accuson, Inc.). No drugs to reduce thrombus formation were administered to the animals.

The results of graft performance in 6 different animals were analyzed. The results are indicated in Figure 5. The implant model described above is an extremely stringent one and leads to rapid graft failure by occlusive clot formation. Normal graft function is denoted by solid bar, and a graft which is failing but still functioning by a striped bar. In the first animal, the control graft and the graft lined with transduced cells secreting enhanced levels of tPA (experimental) failed due to clot formation 24 hours after implant. In all of the other five animals, the graft lined with transduced cells secreting enhanced levels of tPA functioned longer than the graft with cells which had only been mock transduced. This difference varied from 24 hours to several months. These results demonstrate that a therapeutic effect can be achieved in vivo with transduced endothelial cells.

EXAMPLE VI Production of Human Factor VIII from Transduced Endothelial Cells

Endothelial cells were genetically augmented to produce human factor VIII by transducing cells with a retroviral vector, MFG, containing a modified human factor VIII gene (ATCC accession no. 68726). The modified factor VIII cDNA contains all of the coding sequences for the A1, A2, A3, C1, and C2 domains, however the B domain is deleted from amino acids 743 to 1648. The removal of the B domain and the insertion of the modified factor VIII gene into the retroviral vector MFG is described in detail below and depicted in Figure 7.

A full-length cDNA without the 5′ and 3′ untranslated sequences was obtained in a plasmid vector inserted between the restriction sites Nco I (5′) and Xho I (3′). For removal of the B domain, the factor VIII cDNA was subcloned into a plasmid vector in 4 fragments spanning the sequences on both the 5′ and 3′ sides of the B domain. The first fragment of the factor VIII cDNA was subcloned between the restriction sites Sal I and Pst I in the plasmid vector pUC 9. The plasmid vector was cut with Sal I and Pst I and the 5′ phosphates were removed using calf intestinal phosphatase. A 1591 base pair Xho I (nucleotide 7263) to Nde I (nucleotide 5672) fragment, and a 359 base pair Nde I (nucleotide 5672) to Pst I (nucleotide 5313) fragment from the full-length cDNA were isolated and ligated with the Sal I/Pst I digested plasmid vector.

To remove the majority of the sequences encoding the B domain which joins amino acids 742 to 1649 in the same translational reading frame, 4 oligonucleotides were synthesized with a 5′ Hind III site and a 3′ Pst I site covering 168 base pairs. The oligonucleotides extend from the Hind III site at nucleotide 2427 which encodes amino acid 742 followed by amino acid 1649 which is the first amino acid of the activation peptide of the light chain through to the Pst I site at nucleotide 5313. The plasmid vector pUC 9 was digested with the restriction enzymes Hind III and Pst I, and the 5′ phosphates were removed using calf intestinal phosphatase. The oligonucleotides were synth sized as 4 s parate strands, kinased, ann aled and ligated between the Hind III site and the Pst I site of the plasmid vector.

The subcloned Hind III/Pst I oligonucleotide was juxtaposed to the Pst I/ Xho I fragments in a plasmid vector pUC F8. To generate this plasmid, a new polylinker was inserted into a pUC 9 plasmid backbone with the new polylinker encoding the restriction enzyme sites 5′ Sma I-Bam HI-Xho I-Pst I-Hind III-Asp 718-Nco I-Hpa I 3′ used. The plasmid vector was digested with the restriction enzymes Bam HI and Hind III, and the 5′ phosphates were removed with calf intestinal phosphatase. A partial Pst I/ Bam HI digest of the Pst I/Xho I subclone was used to isolate the 3′ terminal factor VIII fragment, and a Pst I/Hind III digest of the subcloned oligonucleotides was used to isolate the heavy and light chain junction fragment. They were ligated into the plasmid vector pUC F8 between the BamHI and Hind III sites.

This subclone containing the factor VIII sequences between nucleotides 2427 and 7205 was digested with Asp 718 and Hind III, and the 5′ phosphates were removed using calf intestinal phosphatase. A fragment encoding factor VIII between the restriction enzyme sites Asp 718 (nucleotide 1961) and Hind III (nucleotide 2427) was isolated and ligated into the plasmid vector to generate a subclone (pF8 3′ delta) containing the factor VIII sequences from nucleotide 1961 through to the translational stop codon at nucleotide 7205.

The construction of the retroviral vector containing the modified factor VIII gene was carried out by inserting the factor VIII gene between the restriction sites Nco I and Bam HI of the retroviral vector MFG. The factor VIII subclone pF8 3′ delta was digested with Sma I

and converted to a BglII site using an oligonucleotide linker. An Asp 718/Bgl II fragment was isolated from the 3' factor VIII subclone, and a 5' factor VIII fragment containing the ATG for initiation of translation was isolated as an Nco I (nucleotide 151)/Asp 718 fragment (nucleotide 1961). The retroviral vector MFG was digested with Nco I and Bam HI, and the 5' phosphates were removed using calf intestinal phosphatase. The factor VIII fragments were ligated into the retroviral vector yielding the final factor VIII retroviral construct, see Figure 6.

The cell line producing the retroviral particles was generated by transfection of the retroviral vector MFG/factor VIII into equal numbers of ecotropic packaging cells Psi CRE and amphotropic packaging cells CRIP as described by Bestwick et al. (Proc. Natl. Acad. Sci. USA 85:5404-5408 (1988)). To monitor the extent of superinfection taking place between the 2 host ranges of packaging cells, the production of biologically active factor VIII was measured using the Kabi Diagnostica Coatest for Factor VIII, Helena Laboratories, Beaumont, Texas and the production of viral RNA was measured by an RNA dot blot analysis. At 21 days post transfection, the mixture of transfected packaging cells was co-cultivated with the amphotropic packaging cell line Psi CRIP-HIS. The CRIP HIS packaging cell line is a variant of the previously described CRIP packaging cell line. The CRIP HIS packaging cell line is identical to the Psi CRIP packaging cell line except that the retroviral envelop gene was introduced into the cell by cotransfection with

pSV2-HIS plasmid DNA, a different dominant selectable marker gene. The packaging cell lines were cultured at a 1:1 ratio for isolation of a homogeneous amphotropic retroviral stock of transducing particles. The superinfection of the amphotropic packaging cell line CRIP HIS has led to the generation of a stable cell line, HIS 19, which produces recombinant retrovirus that efficiently transduce the modified human factor VIII gene. Antibiotic selection of the retroviral producing cell line was not required to isolate a cell line which produces high-titer recombinant retrovirus. The genomic DNA of the cell line has been characterized by Southern blot hybridization analysis to determine the number of integrated copies of the retroviral vector present in the producer cell line. The copy number in the retroviral producing cell line is approximately 0.5, therefore on average 50% of the CRIP-HIS packaging cells contain a copy of the retroviral vector with the modified factor VIII gene. The retroviral vector and the modified factor VIII gene are intact without any deletions or rearrangements of the DNA in the packaging cell line. The copy number of the retroviral vector remains constant with the continuous passage of the retroviral producing cell line. For obtaining the highest titer of recombinant retrovirus, HIS 19 was carried 3 passages in selective histidine minus media followed by 4 passages in completed DMEM media. For the generation of retroviral particles, HIS 19 was seeded at $5x10^5$ - $1x10^6$ cells in a 10 cm cell culture dish. At 48 hours postseeding, approximately 70% confluency, fresh medium (DMEM + 10% calf serum) was added to the plates for

collection 24 hours later as the source of recombinant retrovirus for transduction.

The modified factor VIII gene was transduced into canine endothelial cells isolated from the jugular vein. The endothelial cells were seeded at $3x10^5$ cells per 10 cm. dish in complete M199 medium with 5% plasma derived serum (Equine), 100ug/ml heparin, and 50ug/ml endothelial cell growth factor for 4-6 hours. The cells were then incubated overnight in M199 medium with 5% plasma derived serum, and 100ug/ml endothelial cell growth factor overnight without heparin which adversely affects the efficiency of the transduction process. Cells were exposed to the fresh viral supernatant plus polybrene (8 ug/ml) for 24 hours. After removal of the viral supernatant, the cells were put into M199 medium with 5% plasma derived serum, 100ug/ml endothelial cell growth factor to grow to approximately 70-80% confluence. At that time, the medium was changed to M199 medium with 5% heat inactivated fetal bovine serum (heated at 66°C for 2 hours) , and 50 ug/ml of ECGF. Following a 24 hr. incubation, the medium was collected and assayed for biological active factor VIII by the Kabi Coatest.

With this retroviral producing cell line, between 50% and 75% of the endothelial cells were transduced as determined by Southern blot analysis. The factor VIII gene can be transduced at this frequency with a single exposure to the recombinant retrovirus, and without antibiotic selection of the transduced cells. The transduced endothelial c lls contain an intact copy of the recombinant retroviral genome and th modified factor VIII gene without any

deletions or rearrangements. The rate of production of biologically active factor VIII from the genetically augmented endothelial cells was 400ng/5x10^6 cells/24 hrs.

EXAMPLE 7 In Vivo Transduction of the Endothelium

Using standard stocks of recombinant retrovirus made as described in the previous examples, we have obtained preliminary data demonstrating the in vivo transduction of endothelial cells. The approach is based on the previously published observation (Reidy MA, Schwartz SM, Lab Invest 44:301-308 (1981)) that a defined injury to an artery surface removes a small strip of endothelial cells and this denuded area heals within seventy-two hours by proliferation and in growth of new endothelial cells from the edge of the defect. Cell division is a requirement for effective transduction by recombinant retroviruses and the injury of the endothelium with a wire is one of potentially many methods to induce endothelial cell proliferation. our method uses Reidy's technique of defined injury to induce endothelial cell proliferation, then exposes the proliferating cells directly to supernatants containing recombinant retroviral vectors. Our initial experiments document the ability of this method to successfully transduce endothelial cells in situ, thus potentially avoiding the necessity of tissue culture techniques for the successful introduction of new genetic sequences.

This method requires two surgical procedures, th first procedure injures the blood vessel

surface (here described for the right iliac artery) and induces the proliferation of endothelial cells. The second procedure delivers recombinant retrovirus to the cells undergoing replication on the vessel surface, while preventing the flow of blood from the proximal arterial tree while the proliferating cells are exposed to retroviral particles. For simplicity of performance the procedure is described for iliac arteries.

To demonstrate in vivo gene transfer, we used the marker gene concept published in 1987 (Price J, Turner D, Cepko C. 1987 Proc. Natl. Acad. Sci. USA 84:156160.) with an improved vector based on the α-SGC vector (Figures 2d and 4). The lacZ gene encoding beta-galactosidase was inserted into the α-SGC vector to generate the α-SGC-LacZ vector which is represented in Figure 8. This recombinant construct was transfected into the t Crip packaging cell line and a clone of t Crip cells producing high titers of the α-SGC-LacZ recombinant retrovirus were isolated as described in Example VI. Stocks of the α-SGC-LacZ recombinant retrovirus were used for in vivo transduction.

The experimental animals (rabbits) were anesthetized (ketamine/xylazine), both groins were shaved and prepped, and the animals positioned on an operating table. Through bilateral vertical groin incisions the common, superficial, and profunda femoral arteries were exposed. On the right (the side to be injured) small branches off the common femoral artery were ligat d to insure that outflow from the isolated arterial segment would only occur through the internal iliac artery. If necessary,

the inguinal ligament was divided and the vessel followed into the retroperitoneum to assure complete control of all side branches. The right superficial femoral artery (SFA) was ligated with 3-0 silk approximately 1.5 cm below the profunda take-off, control of the SFA was obtained at the SFA/profunda junction, and a transverse arteriotomy created. A fine wire (the stylet of a 20 gauge Intracath was used), doubled upon itself to provide springiness to assure contact with the vessel wall, was passed up the common femoral and iliac artery retrograde to produce the defined injury described by Reidy et al. The wire was removed, a 20 gauge angiocath was inserted in the arteriotomy and secured to the underlying muscle for immediate access at the next surgical procedure. The incisions were closed in layers and the animals allowed to recover.

Twenty-four hours later a recombinant virus containing supernatant harvested from a crip producer of the α-SGC-LAC-Z vector and supplemented with polybrene to a final concentration of 8 ug/ml was used for in vivo transduction. The animals were again anesthetized and both incisions reopened in a sterile environment. To obtain control of the right iliac vessels above the area that had been injured with no disturbance to the previously denuded right iliac vessel, a #3 FogartyTM balloon embolectomy catheter was inserted through an arteriotomy in the left superficial femoral artery, passed to the aortic bifurcation and the balloon inflated to interrupt blood flow. The right profunda femoris artery was occluded. The supernatant (10 ml) containing the

r combinant retrovirus was introduced by hand injection through the angiocath previously placed in the right SFA. The supernatant flowed in a retrograde fashion from the right common femoral to the right external iliac and into the right internal iliac artery. By leaving the right internal iliac artery open outflow for the supernatant was allowed and a full 10 ml of supernatant could be instilled. In the experiments performed to date the supernatants have been exposed to the vessel wall for periods of four to eight minutes. The catheters from the left and right sides were then removed, hemostasis obtained, and the incisions closed.

Ten to fourteen days later animals were anesthetized prior to sacrifice. After anesthesia and prior to exposure, patency was assessed by direct palpation of the distal vessel. The infra-renal aorta and inferior vena cava were surgically exposed, cannulated, and the vessels of the lower extremity flushed with heparinized Ringer's lactate (2 U/ml) at physiologic pressure (90 mmhg.) . A lethal dose of nembutal was administered and the arteries perfusionfixed in situ in 0.5% gluteraldehyde in 0.1 M cacodylate for 10 minutes. The aorta and both iliac arteries were excised in continuity and rinsed in phosphate buffered saline (PBS) with 1mM MgC_2. The vessels were then stained for lacZ activity by incubation in the x-gal substrate for 1-1.5 hours at 37°C. When the reaction was complete, the x-gal solution was washed away and replaced with PBS.

Two experiments have been completed with this protocol. Both experiments demonstrated successful in vivo transduction as shown by the

in situ xpression of the lacZ gene product in cells on the surface of the artery as visualized by the selective intense blue staining in a cytoplasmic pattern. A line of intensely stained blue cells consistent with the pattern of injury and proliferation described by Reidy et al. is found on the surface of a segment of the external illiac artery injured with a wire, exposed to α-SGC-LacZ recombinant retrovirus, fixed and stained for lacZ activity.

Biological Deposits

On October 3, 1991, Applicants have deposited with the American Type Culture Collection, Rockville, Md., USA (ATCC) the plasmid MFG with the factor VIII insertion, described herein ATCC accession no. 68726, plasmid MFG with the tPA insertion, described herein, given ATCC accession no. 68727, the plasmid α-SGC, described herein, with the factor VIII insertion, given ATTC ascession no. 68728, and plasmid α-SGC with the tPA insertion, described herein, given ATCC accession no. 68729. On October 9, 1991, Applicants have deposited with the American Type Culture Collection, Rockville, MD, USA (ATCC) the plasmid MFG, described herein, given ATCC accession no. 68754, and plasmid α-SGC, described herein and given ATCC accession no. 68755. These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of patent procedure and the Regulations thereunder (Budapest Treaty). This assures maintenanc of a viable culture for 30 years from date of d posit. The organisms will be made available by

ATCC under the t rms of the Budapest Tr aty, and subject to an agreement between Applicants and ATCC which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

Equivalents

Those skilled in the art will recognize, or be able to ascertian using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

Claims

1. A retroviral vector, said vector comprising in operable combination, a 5' LTR and a 3' LTR derived from a retrovirus of interest, and, an insertion site for a gene of interest, wherein said vector does not contain a complete gag, env, or pol gene.

2. A retroviral vector according to Claim 1, said vector further comprising, a portion of a gag coding sequence.

3. A retroviral vector according to Claim 2, wherein said gag coding sequence comprises, a splice donor site and a splice acceptor site, wherein said splice acceptor site is located upstream from said gene of interest.

4. A retroviral vector according to Claim 3, said vector further comprising a gag transcriptional promoter functionally positioned such that a transcript of a nucleotide sequence inserted into said insertion site is produced, wherein said transcript comprises gag 5' untranslated region.

5. A retroviral vector according to Claim 4, wherein said vector does not contain a selectable marker.

6. A retroviral vector according to Claim 5, wherein said vector is MFG having the identifying characterisitics of ATCC 68,754.

7. A retroviral vector according to Claim 1, said v ctor further comprising a gene for

expression ins rted into said insertion site

8. A retroviral vector according to Claim 7, wherein said gene for expression is selected from the group consisting of: a hormone, an enzyme, a receptor, and a drug.

9. A retroviral vector according to Claim 8, wherein said gene for expression is factor VIII or tPA.

10. A retroviral vector according to Claim 1, said vector further comprising, an alpha globin transcriptional promoter.

11. A retroviral vector according to Claim 10, said vector further comprising, a portion of the 5′ untranslated region of the α-globin gene that is naturally joined to said alpha-globin transcriptional promoter.

12. A retroviral vector according to Claim 11, said vector further comprising, an enhancer sequence, wherein said enhancer is not in said 5′ or 3′ LTR.

13. A retroviral vector according to Claim 12, wherein enhancer sequence is located upstream from said transcriptional promoter.

14. A retroviral vector according to Claim 13, wherein said enhancer sequence is a cytomegalovirus enhancer sequence.

15. A retroviral vector according to Claim 14, wherein said vector is α-SGC and having the

identifying characteristics of ATCC No. 68755.

16. A retroviral vector according to Claim 10, wherein said 3' LTR does not contain a functional enhancer sequence.

17. A retroviral vector according to Claim 10, said vector further comprising, a gene for expression inserted into said insertion site.

18. A retroviral vector according to Claim 10, wherein said gene for expression is selected from the group consisting of a hormone, an enzyme, a receptor, and a drug.

19. A retroviral vector according to Claim 18, wherein said gene for expression is factor VIII or tPA.

20. A packaging cell line wherein said cell line has been transfected with a retroviral vector of any of claims 1 and 11.

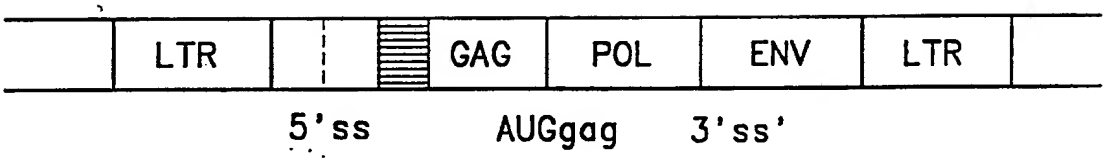


FIG. 1

FIG. 2A
pLJ

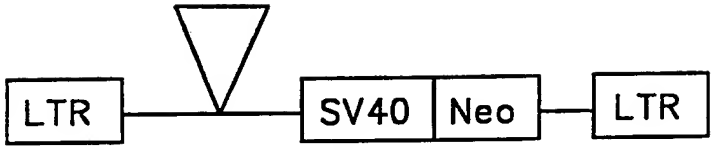


FIG. 2B
pEm

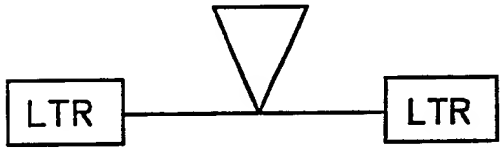


FIG. 2C
MFG

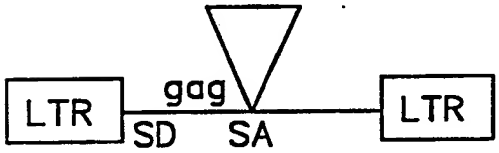
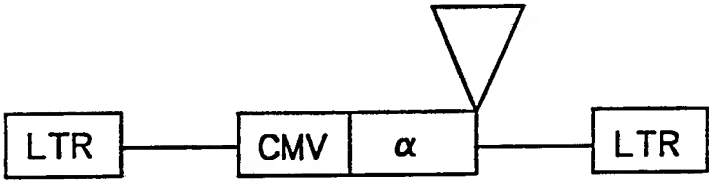
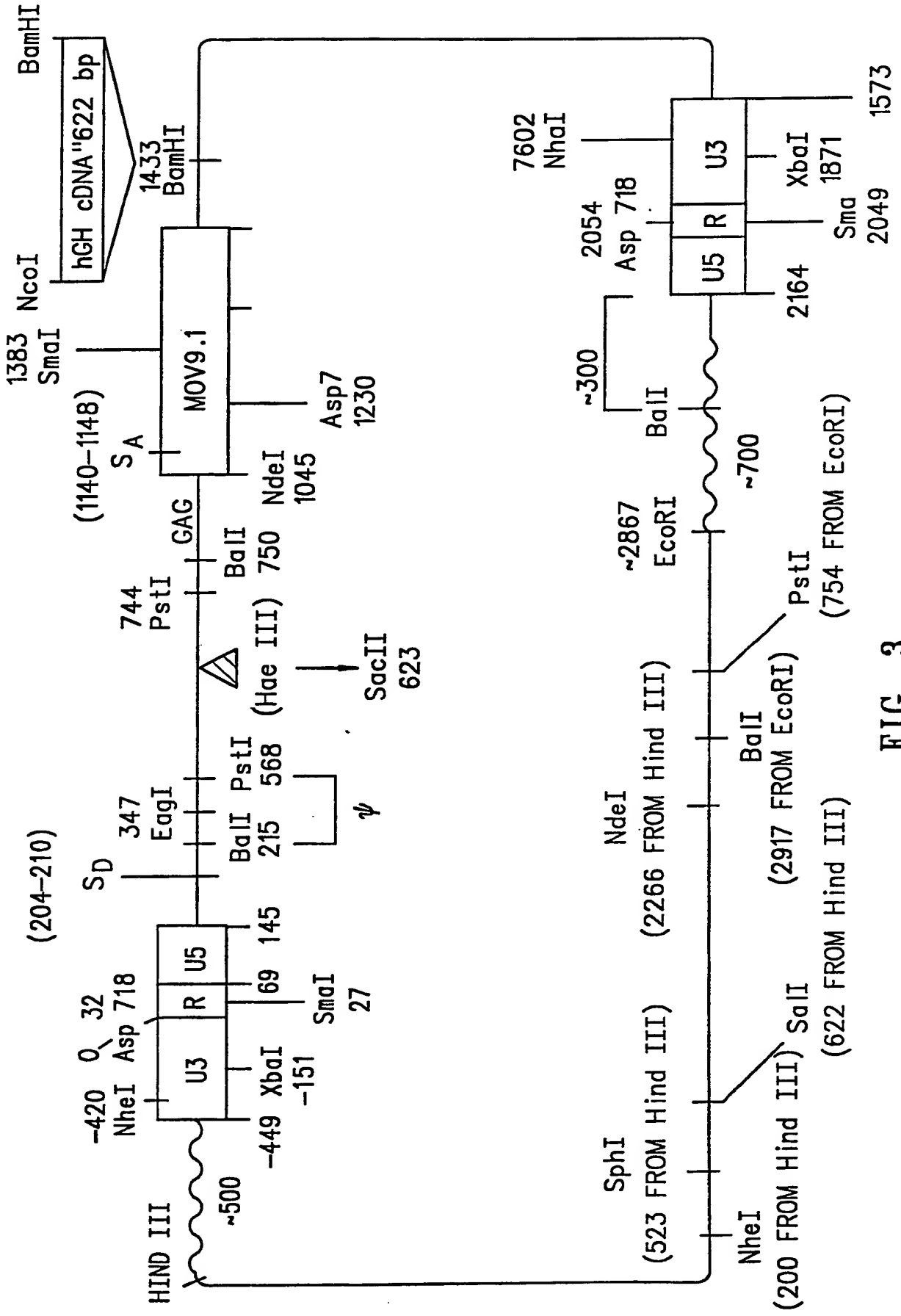


FIG. 2D
αSGC




BamHI
hGH cDNA "622 bp
1433
BamHI
NcoI
1383
SmaI
(1140–1148)
S A
MOV9.1
Asp7
1230
NdeI
1045
GAG
744
PstI
BalI
750
(Hae III)
SacII
623
(204–210)
S D
347
EagI
BalI
PstI
215
568
ψ
145
69
SmaI
27
-420
O
32
NheI
Asp 718
U3
R
U5
XbaI
-151
-449
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HIND III
7602
NhaI
2054
Asp 718
U3
R
U5
XbaI
1871
Sma
2049
1573
2164
~300
BalI
~700
~2867
EcoRI
NdeI
(2266 FROM Hind III)
PstI
(754 FROM EcoRI)
BalI
(2917 FROM EcoRI)
SalI
(622 FROM Hind III)
SphI
(523 FROM Hind III)
NheI
(200 FROM Hind III)

FIG. 3

3/11

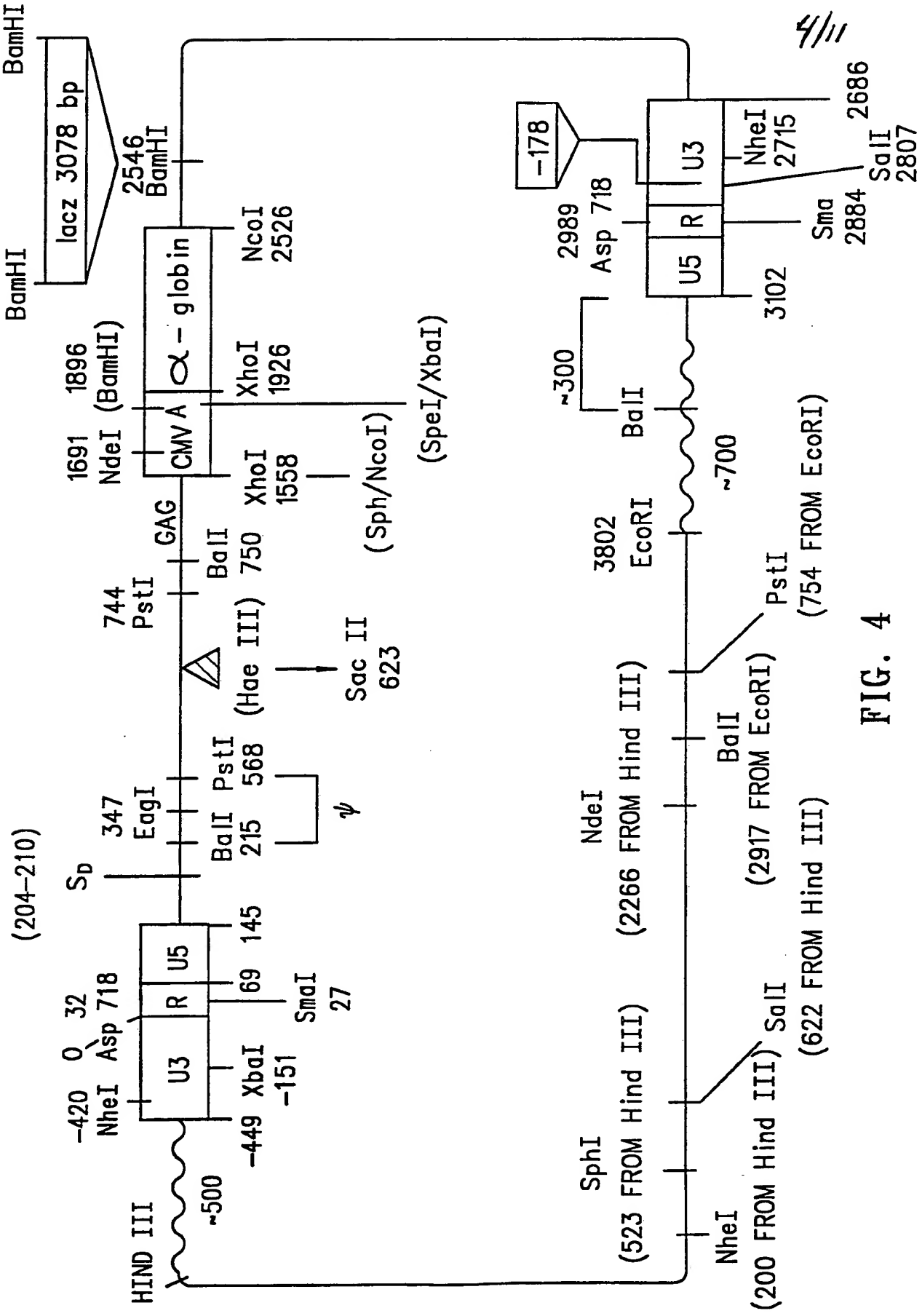
BamHI
lacz 3078 bp
BamHI
2546
BamHI
α - globin
NcoI
2526
1896
(BamHI)
XhoI
1926
1691
NdeI
CMV A
XhoI
1558
(Sph/NcoI)
(SpeI/XbaI)
GAG
BalI
750
744
PstI
(Hae III)
Sac II
623
BalI PstI
215 568
ψ
347
EagI
SD
(204–210)
145
69
SmaI
27
0 32
Asp 718
R
U5
U3
-420
NheI
-449
XbaI
-151
~500
HIND III
-178
2989
Asp 718
~300
BalI
3102
U5
R
U3
NheI
2715
2686
Sma
2884
SalI
2807
3802
EcoRI
~700
NdeI
(2266 FROM Hind III)
BalI
(2917 FROM EcoRI)
PstI
(754 FROM EcoRI)
SphI
(523 FROM Hind III)
SalI
(622 FROM Hind III)
NheI
(200 FROM Hind III)

FIG. 4

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FIG. 5

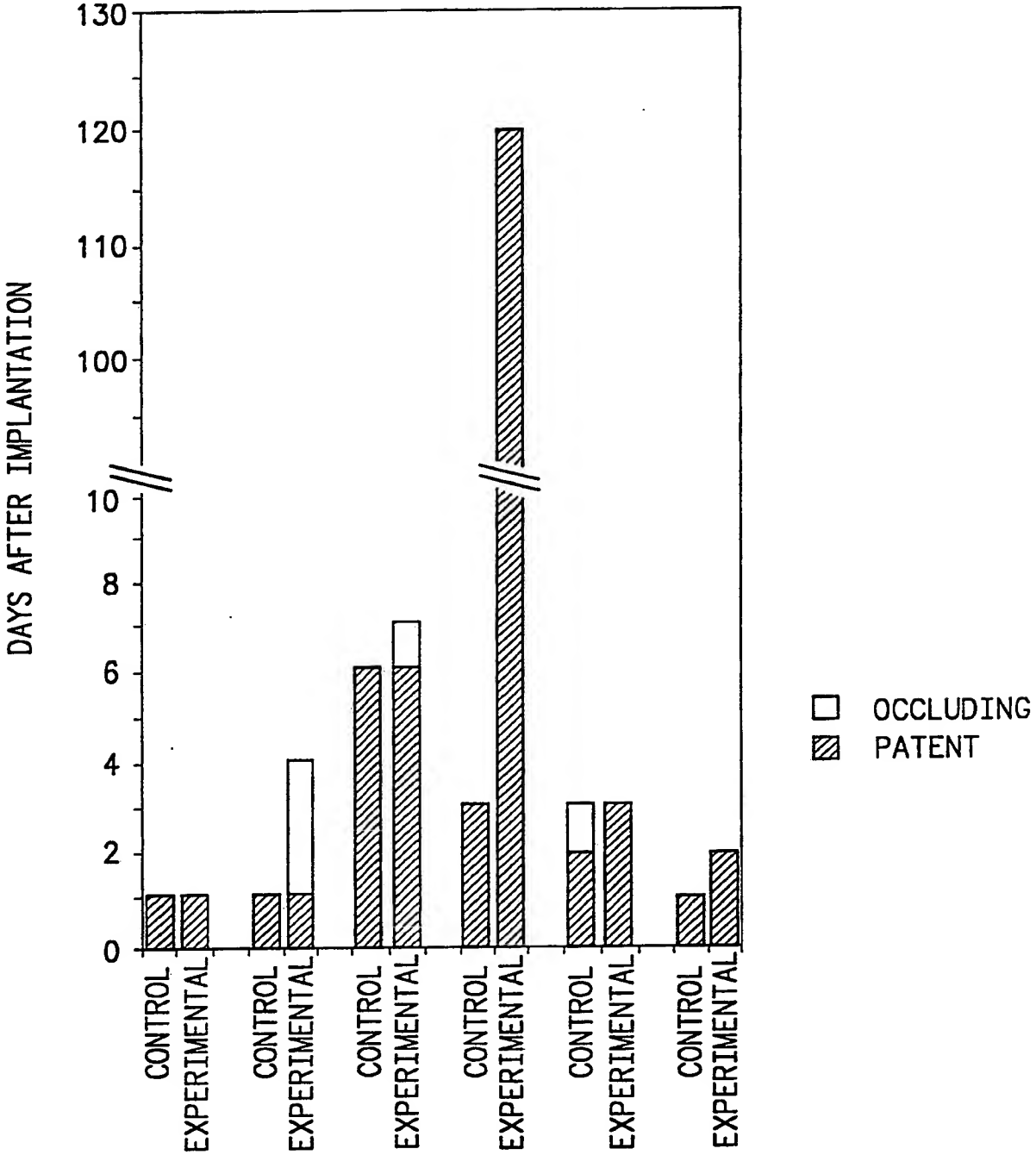

DAYS AFTER IMPLANTATION
130
120
110
100
10
8
6
4
2
0
CONTROL
EXPERIMENTAL
CONTROL
EXPERIMENTAL
CONTROL
EXPERIMENTAL
CONTROL
EXPERIMENTAL
CONTROL
EXPERIMENTAL
CONTROL
EXPERIMENTAL
OCCLUDING
PATENT

FIG. 6A

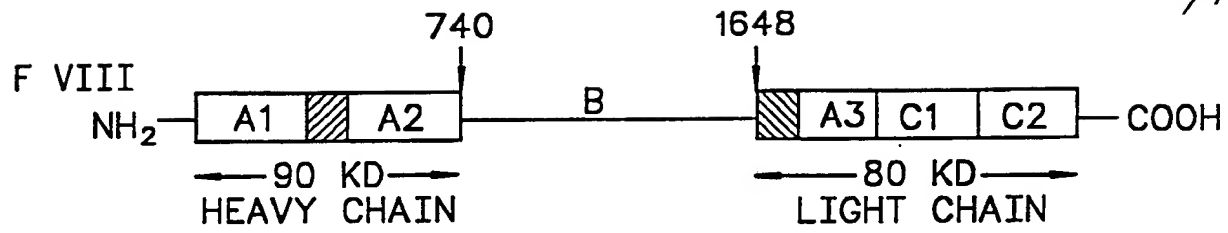


FIG. 6B

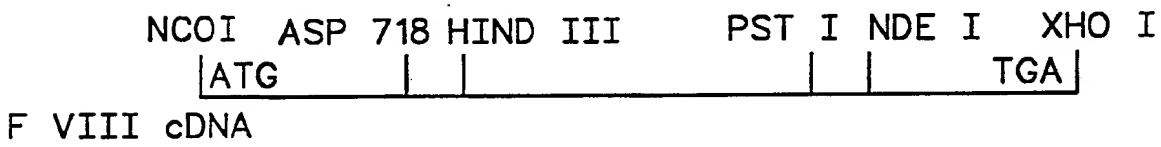
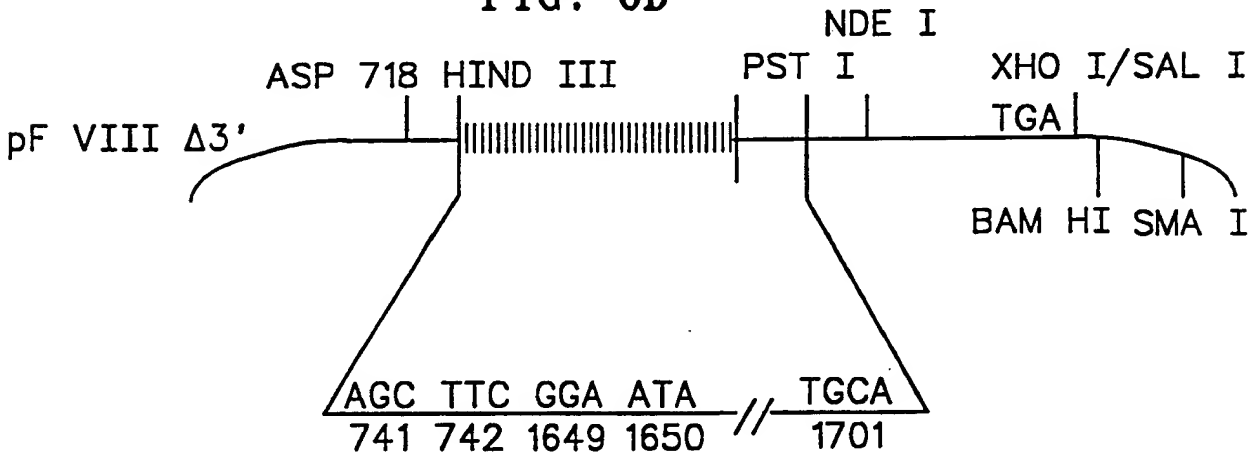


FIG. 6C

FIG. 6D



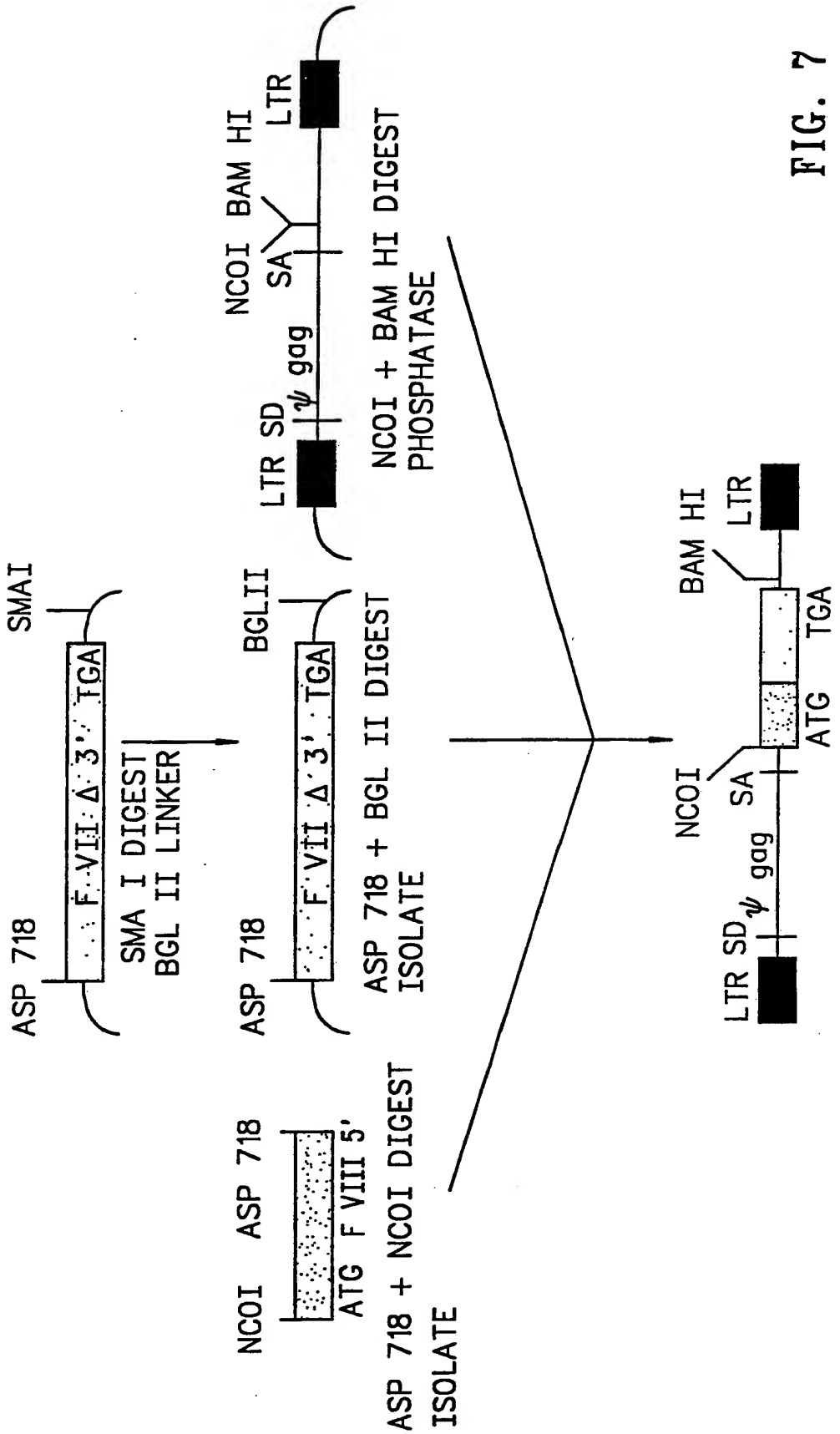

SMAI
ASP 718
F VII Δ 3' TGA
SMA I DIGEST
BGL II LINKER
BGLII
ASP 718
F VII Δ 3' TGA
ASP 718 + BGL II DIGEST
ISOLATE
NCOI BAM HI
LTR
SA
LTR SD
ψ gag
NCOI + BAM HI DIGEST
PHOSPHATASE
NCOI ASP 718
ATG F VIII 5'
ASP 718 + NCOI DIGEST
ISOLATE
BAM HI
LTR
ATG
TGA
NCOI
SA
LTR SD
ψ gag

FIG. 7

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FIG. 8

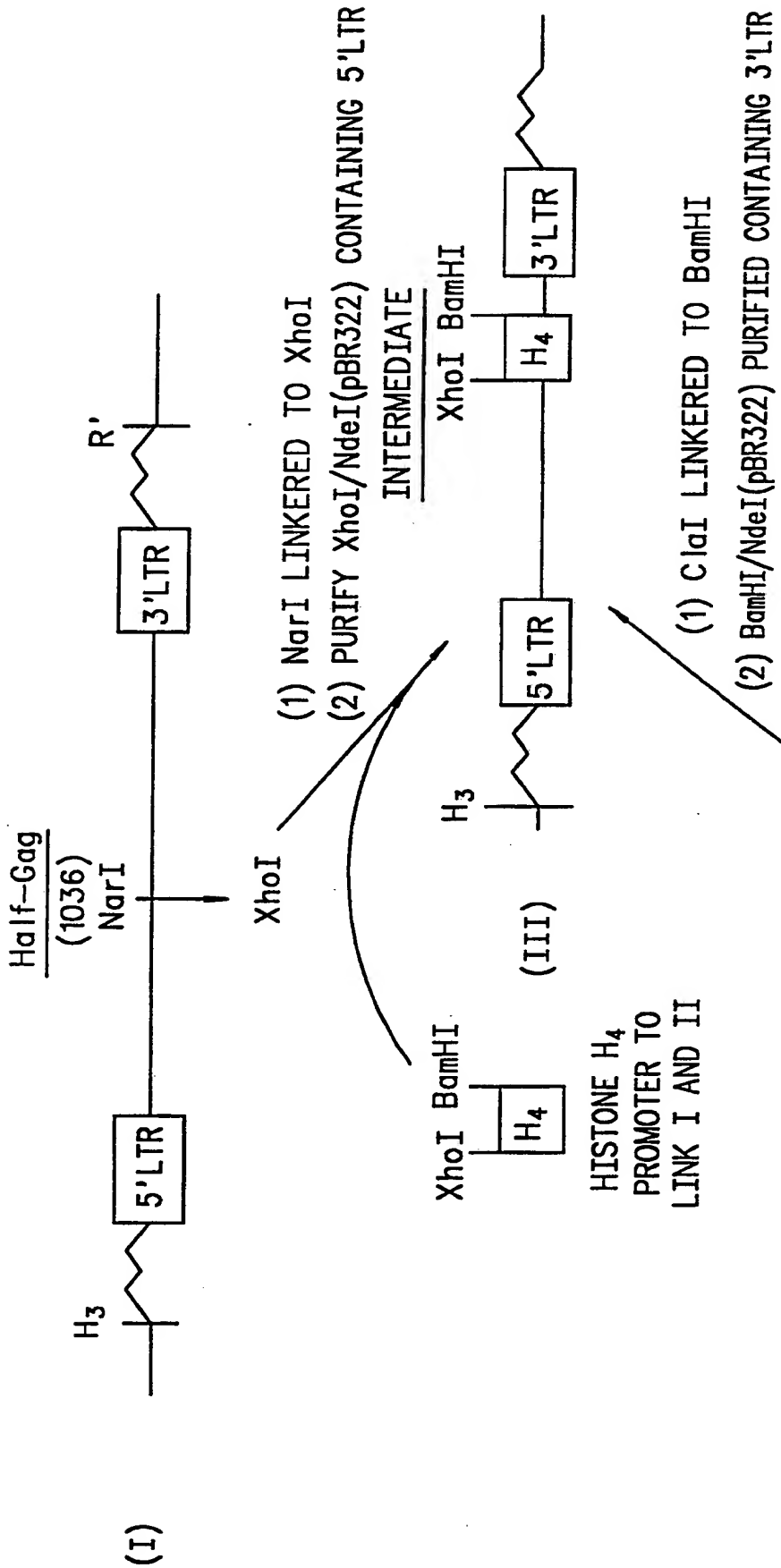
(I)
Half-Gag
(1036)
NarI
R'
3'LTR
5'LTR
H3
XhoI
(1) NarI LINKERED TO XhoI
(2) PURIFY XhoI/NdeI(pBR322) CONTAINING 5'LTR
INTERMEDIATE
XhoI BamHI
H4
3'LTR
5'LTR
H3
(III)
XhoI BamHI
H4
HISTONE H4
PROMOTER TO
LINK I AND II
(1) ClaI LINKERED TO BamHI
(2) BamHI/NdeI(pBR322) PURIFIED CONTAINING 3'LTR
(II)
R1
3'LTR
(7675)
ClaI
BamHI
B2
5'LTR
H3

FIG. 9A

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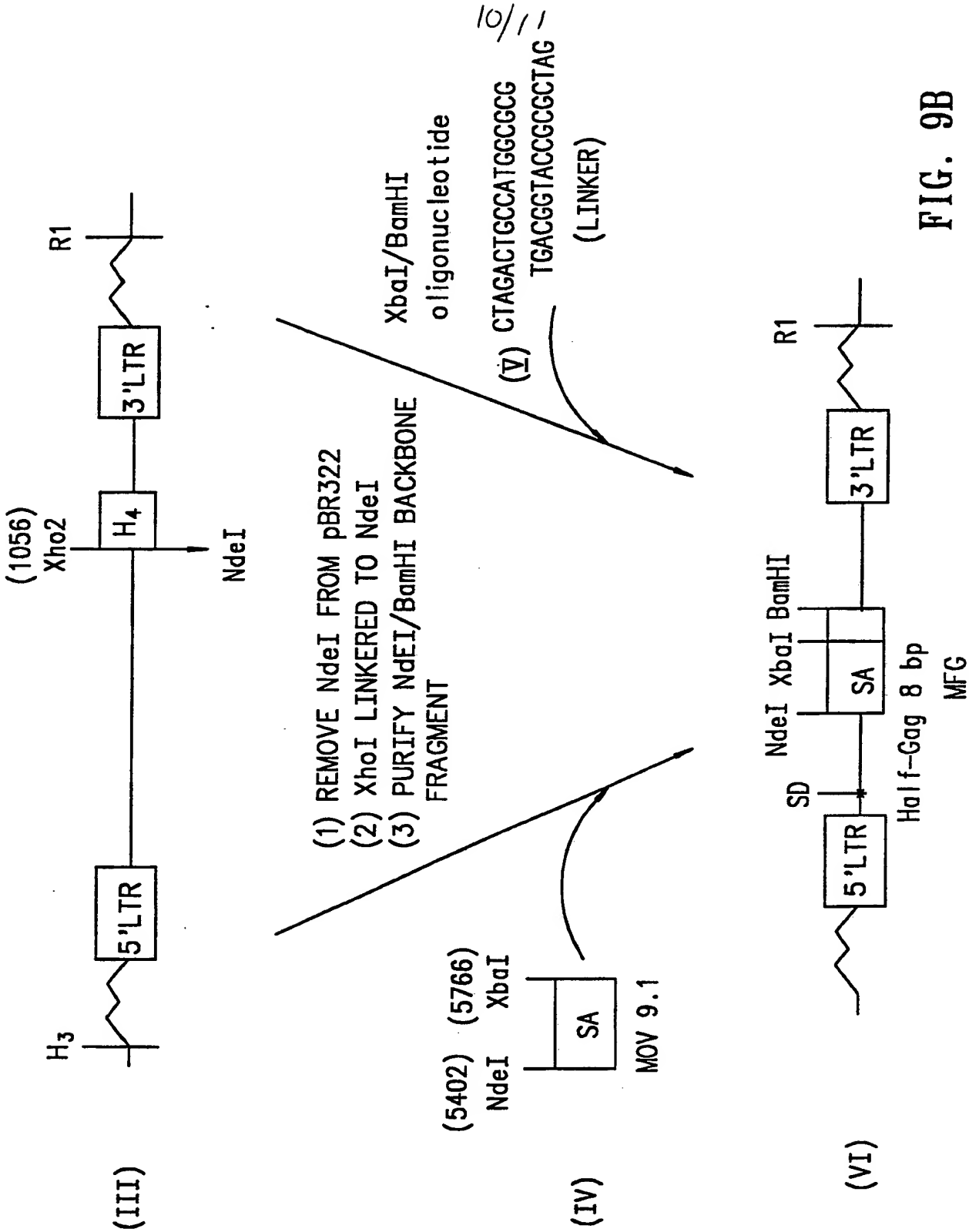
(III)
R1
3'LTR
H4
(1056)
Xho2
NdeI
H3
5'LTR
(1) REMOVE NdeI FROM pBR322
(2) XhoI LINKERED TO NdeI
(3) PURIFY NdeI/BamHI BACKBONE FRAGMENT
XbaI/BamHI
oligonucleotide
(V) CTAGACTGCCATGGCGCG
TGACGGTACCGCGCTAG
(LINKER)
(IV)
(5402) NdeI
(5766) XbaI
SA
MOV 9.1
(VI)
R1
3'LTR
NdeI XbaI BamHI
SA
SD
5'LTR
Half-Gag 8 bp
MFG

FIG. 9B

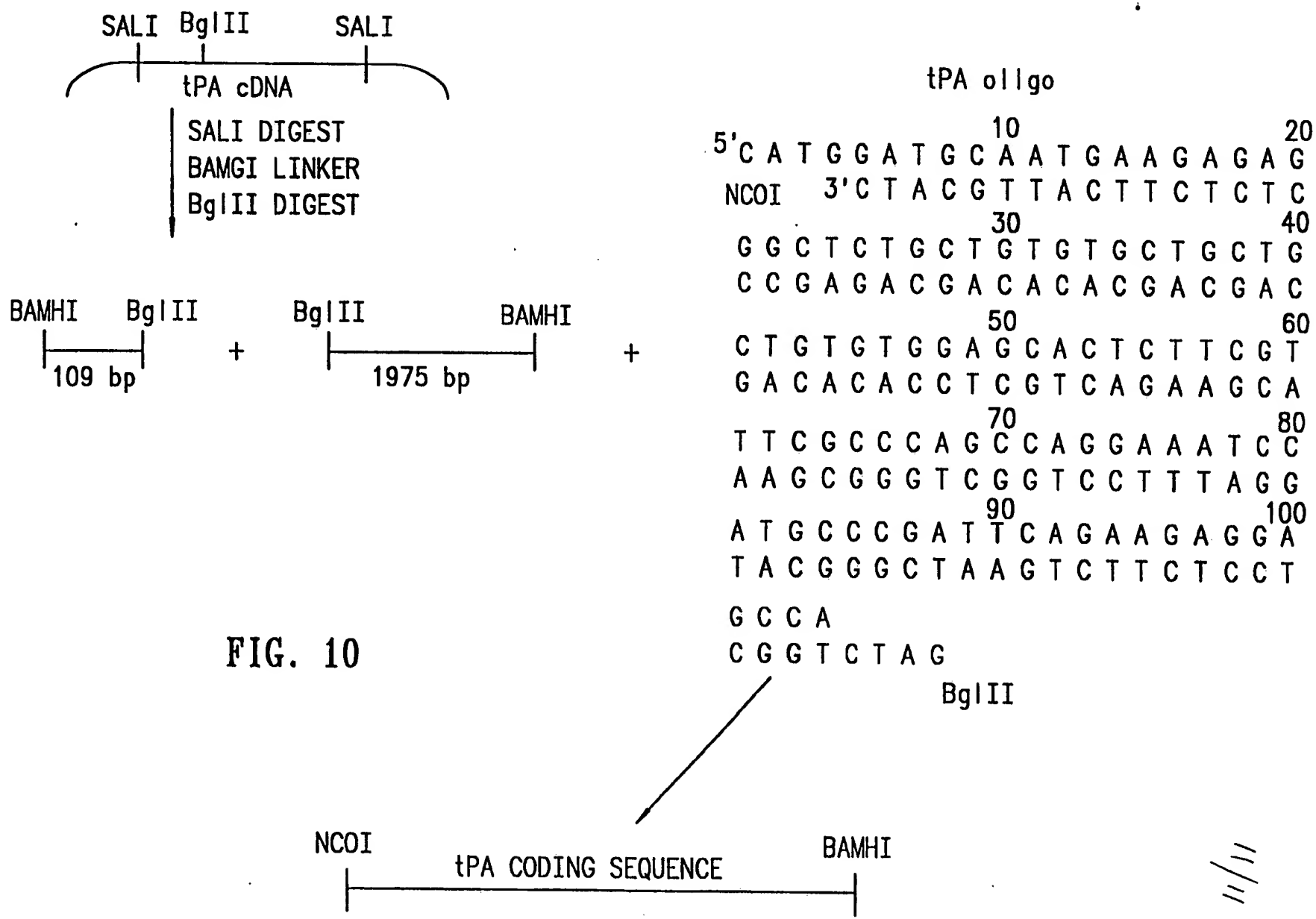

FIG. 10

INTERNATIONAL SEARCH REPORT

Internati nal Application No. PCT/US91/08121

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12N 15/63, 15/86, 15/67, 15/12, 7/01
US CL : Please See Attached Sheet.

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/320.1, 69.1, 69.6, 91, 172.3, 236; 935, 22, 32, 34, 57, 70, 71; 536/27

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[6]

BIOSIS, MEDLINE, APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X/Y	Blood, Volume 75, issued 01 March 1990, Israel et al, "Retroviral-Mediated Transfer and Amplification of a Functional Human Factor VIII Gene", pages 1074-1080, see the entire document.	1, 7-9, 20/17-19
X/Y	Nucleic Acids Research, Volume 18, No. 12, issued 25 June 1990, Morgenstern et al, "Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line", pages 3587-3596, see the entire document.	1-4, 20/5-9
Y	US, A, 4,963,481 (deVilliers) 16 October 1990, see the entire document.	12-15
X/Y	Journal of Virology, Volume 50 No. 1, issued April 1984, Emerman et al, "High-Frequency Deletion in Recovered Retrovirus Vectors containing Exogenous DNA with Promoters", pages 42-49, see the entire document.	1, 10, 11, 17, 18, 20/12-16 and 19

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle r theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
27 FEBRUARY 1992	10 MAR 1992
International Searching Auth rity[1]	Signature of Auth rized Officer[20]
ISA/US	Jacqueline Stone

F rm PCT/ISA/210 (s cond sheet)(May 1986) ß

FURTHER INFORMATI N C NTINUED FR M THE SEC ND SHEET		
X/Y	Journal of Virology, volume 61 No. 5, issued May 1987, Bender et al, "Evidence that the Packaging Signal of Moloney Murine Leukemia Virus Extends into the *gag* Region", pages 1639-1646, see the entire document.	1-3, 7/4-6, 8, 9
X,Y	Molecular and Cellular Biology, Volume 7 No. 10, issued October 1987, Lim et al, "Retrovirus-Mediated Gene Transfer of Human Adenosine Deaminase: Expression of Functional Enzyme in Murine Hematopoietic Stem Cells In Vivo", pages 3459-3465, see the entire document.	1, 20/5

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X/Y	PNAS USA, Volume 85, issued June 1988, Wilson et al, "Correction of the genetic defect in hepatocytes from the Watanabe heritable hyperlipidemic rabbit", pages 4421-4425, see the ntire document.	1-4, 7, 8, 20/5, 6, 9, 12, 13, 15

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

I. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/320.1, 69.1, 69.6, 91, 172.3, 236; 935, 22, 32, 34, 57, 70, 71; 536/27